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A ribozyme transcribed by a ribozyme

Thomas Bentin
Department of Cellular and Molecular Medicine; University of Copenhagen; Copenhagen, Denmark

Prominent current ideas on how life emerged on Earth include an RNA world hypothesis in which RNA performed informational as well as catalytic functions in the absence of both DNA and protein. Demonstration of a self-replicative system based on ribonucleic acid polymers as both information carriers and catalysts would lend support to such a scenario. A pivotal component of this system would be an RNA dependent RNA polymerase ribozyme capable of replicating its own RNA gene. Recent work from the Holliger group at the Laboratory for Molecular Biology in Cambridge has provided synthetic ribozymes[1] that just might foreshadow the future engineering of such self-replicative systems.

Key words: ribozyme, RNA dependent RNA polymerase, In vitro evolution, RNA engineering, transcription

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Correspondence to: Thomas Bentin;
Email: bentin@sund.ku.dk

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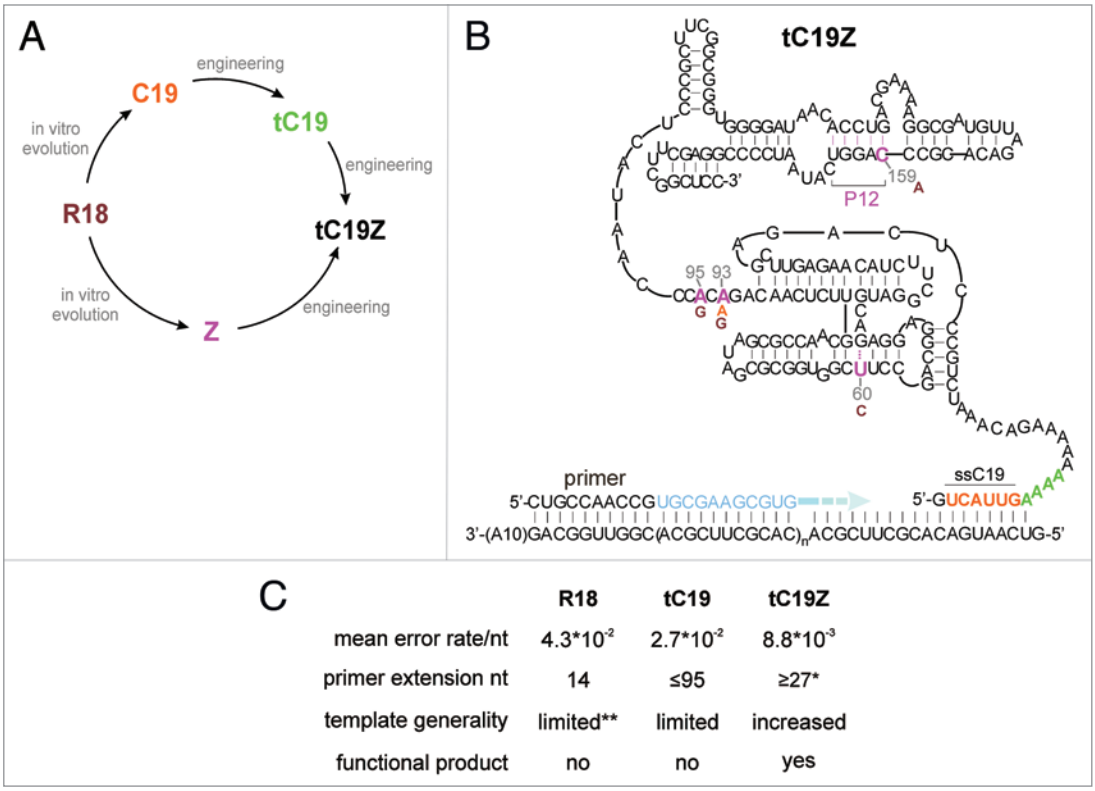
Using R18,[2] a previously described RNA polymerase ribozyme capable of extending an RNA primer hybridized to a complementary RNA template by 14 ribonucleotides (nt), as a starting point, the investigators first evolved/engineered two new RNA polymerase ribozyme lineages (**Fig. 1**); one lineage for enhanced RNA polymerase activity (ribozyme lineage R18 → C19 → tC19), another lineage for improved template sequence generality (ribozyme lineage R18 → Z). The beneficial mutations of Z were subsequently combined with those of tC19 to generate tC19Z showing both enhanced RNA polymerase activity and the ability to transcribe multiple sequence divergent RNA templates. To enable development of these traits, a strategy termed compartmentalized bead tagging (CBT) combining water-in-oil emulsions with bead display, was employed. CBT links the genotype and "phenotype" of a candidate RNA polymerase ribozyme on a single microbead (bead) enabling identification of the encoding DNA gene sequence through isolation of beads showing a selectable property (see **Box 1** for a description of CBT). In vitro evolution yielding ribozyme C19 followed by RNA engineering yielded ribozyme tC19 capable of producing ribonucleotide polymers of 91-95 nt at an efficiency of 0.035% (of total primer) by primer extension using a repetitive sequence RNA template and the four ribonucleoside triphosphates as substrates (**Fig. 1**). Full length RNA products were synthesized with an average error rate per nucleotide position of $2.7*10^{-2}$. Shorter templates, based on the same 11 nt repeat sequence were extended much more efficiently (e.g., 1.5% of primers extended ≥47 nt on an appropriate template). tC19 contains a 5' sequence derived from C19 termed ssC19, the complement of which is present in the selection template used for C19 development. Mutational analyses supported that ssC19 in C19 (and by inference also in tC19 and tC19Z) forms a hybrid with the template 5' analogous to the interaction between the ribosome 16S RNA and the Shine-Dalgarno mRNA sequence in bacteria, as pointed out by the authors.[1] The proximity of the template ssC19 binding site and the primer 3'-OH was an important determinant of activity. Although tC19 carries ssC19 on a flexible 5'-extension (as opposed to the C19 ribozyme which carries ssC19 close to a bulky secondary structure precluding long range primer extension), this organisation would not, apriori, be expected to "reach" a full 95 nt. I would suggest that the template might be "looping out" during primer extension—a common mechanism employed in cellular gene regulation.



	R18	tC19	tC19Z
mean error rate/nt	$4.3*10^{-2}$	$2.7*10^{-2}$	$8.8*10^{-3}$
primer extension nt	14	≤95	≥27*
template generality	limited**	limited	increased
functional product	no	no	yes

Figure 1. RNA polymerase ribozyme development, structure and characteristics. (A) ribozyme lineages showing the descent of tC19Z from R18. (B) Illustration of tC19Z bound via ssC19 to an RNA primer/template complex and active in primer extension (light blue). Brown, orange, green and purple nucleobases derives from R18, C19, tC19, and Z, respectively (same color code as in A). As regards the specific selected sequence elements, the ssC19-A4 combination facilitates template binding and RNA polymerase activity on longer templates; C60U provides a potential wobble base pair in the catalytic core (dashed line), and enhances RNA polymerase activity; G93A and G95A are compensatory mutations reducing a need for a short "stem" structure, which in R18 is provided by an RNA oligonucleotide in trans; A159C, located in the processivity domain, enables formation of the P12 stem by allowing G133:C159 base pairing. (C) Ribozyme characteristics. *The primer extension capability of tC19Z in terms of product length remains unknown because it was not tested with the long template yielding 95 nt products with tC19. **Template generality: according to Johnston et al. (2001),[2] R18 worked with all primer/template complexes tested (producing RNA oligomers up to a maximum of 14 nt) and is therefore a general RNA polymerase. However, both R18 and tC19 remained template sequence dependent according to Wochner et al. (2011),[1] and are therefore indicated as "limited" in the figure as opposed to tC19Z, which showed increased template sequence generality.

tC19 showed a template sequence dependence incompatible with it being a general RNA polymerase ribozyme. Selection for template sequence generality was done starting with a $7.5*10^{7}$ member library of randomized R18 variants. This library was subjected to CBT using two sequence distinct RNA primer extension templates, which yielded Z, a ribozyme harbouring four point mutations that together decreased template restrictions. These mutations were combined with those of tC19 thus generating tC19Z. tC19Z showed a further reduced average error rate of $8.8*10^{-3}$ per nucleotide position, and also proved superior to R18, C19, Z and tC19 ribozymes in terms of RNA polymerase activity and generality (although its potential in producing RNAs ≥ 95 nt was not reported). tC19Z was used to produce a 24 nt hammerhead minizyme by primer extension of a 5' fluorescein isothiocyanate (FITC) labeled primer. When challenged with its substrate, this FITC labeled RNA minizyme proved cleavage proficient to the same extent as the FITC-labeled control hammerhead ribozyme made by solid phase synthesis. These results demonstrate replication combined with conservation of function—a probable key feature of an ancient RNA based self-sustained system.

The new ribozymes described differ from present day protein RNA polymerases in several respects, as well as lacking protein. Cellular RNA polymerases are efficient showing both a high propensity to remain associated with its transcript in the elongation complex (processivity) and rapid elongation rates of typically ~200 nt/s (at 37°C) in the case of bacteriophage RNA polymerases. In comparison, tC19 synthesized RNAs of maximally 95 nt in a typical period of 24 h corresponding to $\sim 1*10^{-3}$ nt/s (at 17°C). Cellular RNA polymerases also show a higher fidelity (in part due to proofreading) with an estimated error rate of $<10^{-5}$ per nucleotide position for bacterial and eukaryotic RNA polymerases.[3] Finally, cellular RNA polymerases initiate RNA synthesis at the site of template recognition (the promoter), do not require a primer, and employ a double-stranded DNA template. Perhaps the closest parallel to the described ribozymes is found among RNA vira that express RNA dependent RNA polymerases, some of which utilize a primer.

A truly self-replicating RNA polymerase ribozyme based system would require that

Box 1. Compartmentalized Bead Tagging CBT involves immobilization of ≤1 ribozyme encoding DNA fragment, hereinafter designated "DNA gene", per bead. The DNA gene is subjected to run-off transcription using phage T7 RNA polymerase. Due to water-in-oil emulsification, and using additional ingenious tricks, the candidate RNA polymerase ribozyme (produced in thousands of copies) binds to the parent bead that contain its encoding DNA gene, only. To provide a substrate for the RNA polymerase ribozyme candidates, the beads are pooled, and unbound material (incl. T7 RNA polymerase and unbound ribozyme) is washed off; next an RNA primer is immobilized to the beads in yet more copies (≤120.000/bead), and a complementary longer "template" RNA is annealed to the bound primer. Using these beads (decorated with a single gene, 3000 ribozyme copies, and potentially even more primer/template complexes), yet an emulsion is made. The clonal ribozymes are liberated within their emulsion drop enabling selective primer extension on the parent bead. The beads are again pooled and the RNA template and the ribozyme are washed off. The primer extension product, which remains bound to the parent bead, is subjected to rolling circle amplification (RCA). In RCA, a single-stranded DNA minicircle is annealed to the primer extension product that, in turn, functions as the primer for enzymatic amplification. The RCA product is detected using complementary fluorescent probes. The beads are sorted using fluorescent activated cell sorting (FACS), and ribozyme genes in the selected fluorescent bead pools are PCR amplified and subjected to additional rounds of CBT as desired. In effect, beads showing increased fluorescence harbour DNA genes encoding RNA polymerase ribozymes with enhanced activity. Using CBT, up to 10^5-fold enrichment of an active R18 ribozyme spiked into a surplus of inactive R18 mutant pool was achieved supporting the utility of CBT for selection of ribozyme RNA polymerases.

the ribozyme fully replicate its own RNA gene (unlike present day DNA dependent RNA polymerases that do not transcribe the promoter region), including any ssC19 type docking sites on the template. The encoding RNA gene would have to be relatively unstructured to allow efficient primer extension of the entire sequence, and there would need to be a mechanism for strand separation to enable replicative turnover. Even if these requirements can be met, the present ribozymes capable of maximally synthesizing a 95 nt RNA transcript would still fall short of copying their own gene of 187 nt thus precluding their immediate use for self-sustained replication. Recognising that complex requirements remain to achieve a fully operative self replicating system, it is appropriate to praise the achievements of Wochner et al. 2011,[1] which on the tenth anniversary of the birth of R18[2] have provided a great technological leap and renewed interest in a fascinating and challenging area.

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